RAPID AMINOLYSIS OF BENZOYLTHIONOCHOLINES, APPLIED IN PEPTIDE SEQUENCE ANALYSIS. RELEVANCE TO PHYSIOLOGICAL PROPERTIES OF SULPHUR ANALOGUES OF CHOLINE ESTERS

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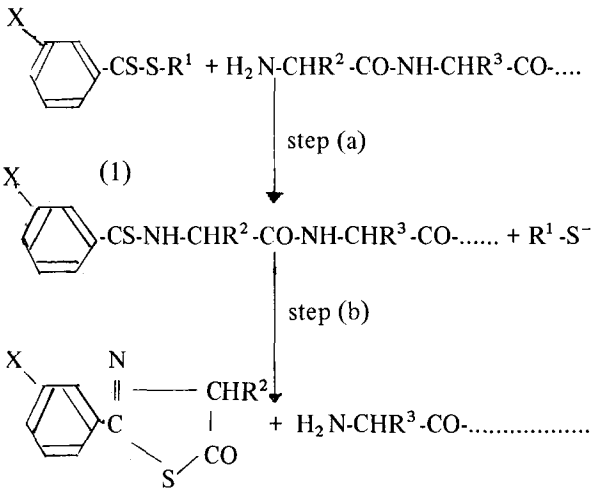
1. Introduction

A method [1] for peptide sequence determination, using the terminal *N*-thiobenzoyl derivative of the peptide, is closely related to the Edman stepwise degradation, [2] but differs in several respects so that improvements to current methods [3] of automated Edman sequence determination might be developed [1a].

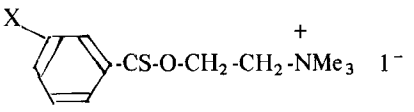
The two steps in a cycle of an Edman degradation are (a) reaction of the terminal amino group of the peptide with phenyl isothiocyanate, and (b) cleavage of the resulting *N*-phenylthiocarbamoyl peptide to give a shortened peptide and a 2-anilinothiazolone. The thiazolone is readily rearranged into the isomeric phenylthiohydantoin, identification of which defines the *N*-terminal amino acid residue of the peptide. The steps ((a) and (b) in Scheme) illustrating the analogous method using *N*-thiobenzoyl derivatives [1a] differ in using a dithioester *cleavage* reaction in aqueous solution for step (a) rather than a nucleophilic *addition* reaction to phenyl isothiocyanate in mixed solvents as in the Edman method, and in the formation in step (b) of a 2-phenylthiazolone which is not susceptible to rearrangement.

With yields less than quantitative in steps (a) and (b), and with the accumulation of artifacts from side-reactions, [4] the results from an extended Edman sequence determination become progressively more ambiguous, [3,5] and there is scope for improvements so that long sequences can be tackled with confidence. The present paper describes improved methodology for step (a) in the method using thiobenzoyl derivatives, when the dithioester (1; $R^1 = CH_2\text{-}CO_2H$) is replaced



Scheme

by the thionester[6] (2) which embodies the β-trimethylammonioethyl group capable [7] of facilitating aminolysis of the neighbouring thionester function through anchimeric assistance, and also allowing ion-exchange removal of excess reagent (2) and of choline from reaction mixtures.



(2)

The results of a kinetic study are reported, showing that *m*-nitrobenzoylthionocholine iodide (2; X = NO_2) is capable of the quantitative *m*-nitrothiobenzoylation

of a peptide within 10 min at pH 9.2 in aqueous solution at room temperature. On this basis, several cycles of a sequence determination can be performed on a peptide within a day.

2. Results

2.1. *Benzoylthionocholines (2)*

(i) Carboxymethyl dithiobenzoate (1; $R^1 = CH_2\text{-}CO_2H$) [8] or a ring-substituted analogue [9] was added to a soln of β-dimethylaminoethanol (20 equiv.) in tert-butanol containing 2 equiv. *tert*-BuONa, and the mixture was heated under reflux during 15 min. Ethyl acetate (50 ml) and water (50 ml) were added, and the dried ($MgSO_4$) extracts were concentrated, filtered through neutral alumina, and evaporated. Iodomethane was added to the residual oil in soln in benzene and ether; the choline ester iodide (2) separated from the soln during 2 days, and was collected and washed with ether. Overall yields were approx. 20%. The *m*-nitro-compound (2; X = NO_2) had m.p. 166–8°C (found: C,36.2; H,4.45; N,6.95; S,7.95; I,32.4; $C_{12}H_{17}N_2O_3SI$ requires: C,36.35; H,4.3; N,7.05; S,8.1; I,32.05%).

(ii) Higher yields of (2) were obtained using tetrahydrofuran as reaction medium, and sodium hydride as base [10].

2.2. *Thiobenzoylation of peptides using m-nitrobenzoylthionocholine iodide* (2; X = NO_2)

Solutions of the peptide (1 μmol) and (2; X = NO_2; 0.002 g, 5 μmol) in 200 μl buffer (0.36 M in Et_3N, 0.2 M in pyridine, and 0.25 M in acetic acid; pH 9.2) were mixed and set aside at 25°C during 15 min. The mixture was diluted to approx. 1 ml with water, and was filtered through a 3 × 1 cm column of SP-Sephadex C25 which had been pre-treated first with buffer, then with water. The eluate, and water washings (2 ml), were evaporated to dryness, giving the *N*-(*m*-nitrothiobenzoyl)peptide; cleavage with trifluoroacetic acid (step (b) in Scheme) and t.l.c. analysis showed that the amino group of the peptide had been completely *m*-nitrothiobenzoylated.

2.3. *Kinetic study of thiobenzoylation of peptides using thionbenzoates* (2)

The progress of thiobenzoylation in mixtures prepared as above was followed through the reduction in optical density at 430 nm (λ_{max} of thionbenzoates 405 nm in H_2O; $\lambda_{inflection}$ of *N*-thiobenzoylpeptide approx. 360 nm). Second order rate constants for the thiobenzoylation of glycyl-L-leucine using the dithioester (1; $R^1 = \text{-}CH_2\text{-}CH_2\text{-}N^+Me_3I^-$ and its *m*-nitro-substituted analogue were 1.2 and 4.7 litres mol^{-1} min^{-1} respectively, and 39.9 and 92.3 litres mol^{-1} min^{-1} respectively for the thionbenzoates (2; X = H and X = NO_2). Blank experiments showed that the choline esters (2) underwent no appreciable hydrolysis during a kinetic run at pHs between 8.4 and 9.6.

2.4. *Cleavage of N-thiobenzoylpeptides (step (b) in* Scheme)

Trifluoroacetic acid was added to the *N*-thiobenzoylpeptide; after 2–3 min. the solution was evaporated at room temp in a stream of N_2.

2.5. *Identification of cleavage products from N-thiobenzoylpeptides*

2.5.1. Gas-liquid chromatography

Bis(trimethylsilyl)acetamide or -trifluoroacetamide (100 μl) followed by acetonitrile (100 μl) was added to the evaporated cleavage mixture. A sample of the silylated thiazolone and peptide mixture obtained in this way was analysed by g.l.c. (Pye 104, 1ft column, 2% OV1 silicone oil on 80–100 mesh Supasorb pretreated with AW-HMDS) at operating temperatures 190–240°C, when the silylated thiazolones were characterised by retention times between 3–12 min*.

2.5.2. Thin-layer chromatography

The silylated cleavage mixture was dissolved in a mixture of ether (5 ml) and buffer (0.2 ml), and the ether soln was extracted with a further 0.2 ml buffer. A portion of the buffer containing 20 nmol shortened peptide was studied by t.l.c. on silica gel using *n*-BuOH:AcOH:H_2O (4:1:1) for development. After examination under u.v. light (254 nm), the plate was revealed with ninhydrin.

* Details of mass-spectrometric identification of silylated cleavage products will be reported elsewhere (G. C. Barrett, J. R. Chapman, and P. H. Leigh).

2.6. *Sequence analysis of peptides*

(i) Leucyl–glycyl–phenylalanine. The peptide was treated as above(successive *m*-nitrothiobenzoylation, cleavage, and removal of the 4-isobutylthiazolone); repetition of the *m*-nitrothiobenzoylation and cleavage steps gave phenylalanine, complete conversion in both cycles being established by t.l.c, using both glycyl–phenylalanine and phenylalanine standards at 20 nmol concentrations. All manipulations (except the development of the thin-layer chromatogram) were completed within one hour. Since a faint trace of glycyl–phenylalanine remained after the second cycle, in one of several repetitions of this particular sequence analysis, a time of 20 min can be recommended for the *m*-nitrothiobenzoylation step as a routine to ensure essentially complete conversion.

(ii) Sequence analysis of Gly–Phe–Ala, Ala–Leu–Gly, Gly–Leu–Ala, Met–Ala–Ser, and Val–Ala–Ala–Phe was accomplished in the same way; present studies include peptides containing other representative protein amino acids.

2.7. *Note on the study of physiological properties of sulphur analogues of choline esters*

Substantial differences have been reported [6] between the physiological properties of benzoylcholine and its thiono analogue (2; X = H); interpretation of structure–activity relationships should take into account the extraordinarily large rate of aminolysis and low rate of hydrolysis established in the present work for benzoylthionocholines (2).

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